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RECOMBINANT MULTIVALENT VIRAL VACCINE

This application is a continuation of co-pending U.S. application serial no. 08/552,369 filed on November 3, 1995, which is a continuation-in-part of United States application Serial No. 08/190,789, filed January 27, 1994, now abandoned, which is a continuation of United States application Serial No. 726,609, filed July 9, 1991, now abandoned, the disclosures of which are incorporated herein by reference.

Field of the Invention

The present invention relates generally to the production and use of a recombinant viral vector as a multivalent vaccine in the protection of felines against infection by various viral pathogens of felines. More particularly, the present invention relates to a recombinant multivalent vaccine formed by inserting multiple genes such as a feline panleukopenia virus (FPV) gene, a rabies virus gene, and/or a feline calicivirus (FCV) capsid protein gene each operably linked to a promoter, into a raccoon poxvirus (RCNV) for expression.

Background of the Invention

1. Feline panleukopenia virus biology and pathology

Feline panleukopenia virus (FPV) is a highly contagious viral disease of domestic cats and exotic cats. The virus is transmittable to susceptible cats by contact with body secretions and excretions of infected cats during the acute phase of infection in which virus is shed. Aerosol transmission, and transmission by

insect vectors can also occur. The virus infects and destroys actively replicating cells in lymph nodes, and hematopoietic and gastrointestinal tissues of felines thereby causing sudden onset of symptoms including fever, anorexia, leukopenia, vomiting and diarrhea. In particular, FPV causes severe clinical illness in young kittens with high morbidity and mortality. A marked drop in total leukocyte count by day 4 to 6 after infection is the prominent indicator of FPV infection. Disease caused by infection with FPV has been described as feline parvovirus, feline panleukopenia, infectious enteritis, viral enteritis, cat "distemper", granulocytosis, cat plague, and cat fever.

FPV is a small, single stranded DNA virus, a parvovirus, that requires rapidly multiplying cells for DNA replication. The genome of FPV is a linear, single stranded DNA of about 5 kilobases in size that encodes three structural proteins: a large 80-85 kilodalton (kd) protein ("VP1") comprising 10% to 15% of the viral protein; a medium size protein of 64-67 kd ("VP2"); and a part of the VP2 protein which is converted to a 60-64 kd protein ("VP3") by proteolytic cleavage. The three proteins physically form a nested set of proteins within which the viral DNA is enclosed. FPV is very closely related to canine parvovirus (CPV) and mink enteritis virus both on the protein and amino acid level (Tratschin et al., 1982, *J. Gen. Virol.,* 61:33-41; Truyen et al., 1994, *Virology* 200:494-503; and Truyen et al., 1994, *J. Virol.* 66:5399-5408). The high cross-reactivity between CPV and FPV indicated their antigenic similarities and the possibility of mutual neutralization and protection between the two viruses.

2. Rabies virus biology and pathology

Rabies virus is a member of the genus Lyssavirus in the family Rhabdoviridae, and contains an unsegmented negative stranded RNA genome. Of the five known viral structural proteins, the rabies virus transmembrane glycoprotein G plays a critical role for the induction and binding of the virusneutralization antibodies and the stimulation of T cell-mediated immunity (Lafon et al., 1983, *J. Gen. Virol.* 64:843-851; Lafon et al., 1985, *J. Gen. Virol.* 66:2125-2133; Wiktor et al., 1973, *J. Immunol.* 110:269-276; Wiktor et al., 1984, *Dev. Biol. Stand.* 57:199-211; Wiktor et al., 1984, *Proc. Natl. Acad. Sci. U.S.A.* 81:7194-7198). Also, the arginine, at position 333 in the glycoprotein amino acid sequence, is essential for the integrity of at least one antigenic determinant and for the ability of rabies virus to produce a lethal infection in adult mice (Dietzschold et al., 1983, *Proc. Natl. Acad. Sci. U.S.A.* 80:7074). Initial symptoms of rabies virus infection include fever, and malaise. The disease progresses rapidly to symptoms including agitation, convulsions, and coma; and eventually, if untreated, the infected animal may die from organ failure.

3. Recombinant veterinary vaccines

In the art of veterinary vaccines, purified recombinant VP2 protein has been used as an immunogen for protecting dogs against infection by canine parvovirus (Wood et al., U.S. Patent No. 4,971,793). In terms of viral vaccine vectors, vaccinia virus recombinants have been constructed with insertion of a respective gene encoding either feline infectious peritonitis virus (FIPV) spike protein, membrane

glycoprotein, or nucleocapsid protein (Vennema et al., 1990, *J. Virol.* 64:1407-1409; Vennema et al., 1991, *Virology* 181:327-335). Immunization with such a vaccinia virus recombinant appeared to be of little or no value in the protection of vaccinated kittens against challenge with FIPV. Thus, vaccinia virus vectors do not appear to be a good choice for constructing feline recombinant vaccines because: (a) vaccinia virus/viral vectors did not elicit protection or detectable virus neutralization antibodies (Vennema et al., 1991, *supra;* Scott, 1988, *Conf. Res. Workers Anim. Dis.* 69:60); and (b) of concerns of introducing recombinant vaccinia virus for veterinary or human use, particularly because of rare side effects associated with vaccinia virus immunization.

In contrast, a raccoon poxvirus (RCNV) recombinant vector containing the gene encoding rabies virus surface glycoprotein G, has been used successfully to induce immunity in raccoons which is protective against subsequent challenge with raccoon rabies virus (Esposito et al., 1988, *Virology,* 165:313316). However, the investigators report that the complete host range of RCNV is not known. A recombinant RCNV containing the gene encoding FPV VP2 protein ("recombinant RCNV/FPV") was recently constructed by inserting the VP2 protein gene into a vaccinia expression vector, and then recombining the insertion into the thymidine kinase (TK) gene of RCNV. In a vaccine trial, all cats immunized with the RCNV/FPV were fully protected against subsequent FPV challenge, and showed high titers of FPV viral neutralization antibody (U.S. Patent Application Serial No. 08/190,789 assigned to the assignee of the present invention). Presently, there are no reports of the design of a functional multivalent RCNV-vectored

vaccine, and its use for inducing protective immunity in felines.

Summary of the Invention

A novel raccoon poxvirus (RCNV) recombinant vaccine against both feline panleukopenia virus (FPV) and rabies virus was developed based on the homologous thymidine kinase gene between vaccinia virus and RCNV. The infectious recombinant virus (RCNV/FPV/RAB) carried both FPV VP2 and rabies G protein genes, each operably linked to a promoter. Vaccine trials using RCNV/FPV/RAB induced strong immune responses in cats to both FPV and rabies virus. Cats immunized with RCNV/FPV/RAB were fully protected against subsequent FPV challenge. Viral neutralization antibody titers for both FPV and rabies virus were sufficient to protect cats from the related virulent virus infection or challenge. In another embodiment, a novel raccoon poxvirus multivalent recombinant vaccine can be developed based on an insertion vector being constructed to have homologous hemagglutinin (HA) gene sequences which flank one or more inserted genes (such as FCV capsid protein gene), and thus allowing recombination into the hemagglutinin gene sequences of raccoon poxvirus. Thus, the methods and compositions of the present invention provide the basis for producing novel multivalent recombinant vaccine vectors for felines. Using the methods according to the present invention, a multivalent recombinant raccoon poxvirus could have the following insertions: one or more exogenous genes recombined into both the raccoon poxvirus TK and HA gene sequences; more than one exogenous gene recombined into the raccoon poxvirus TK gene sequences; and more than one exogenous

gene recombined into the raccoon poxvirus HA gene sequences.

Accordingly, it is one object of the present invention to provide a method for inserting more than one exogenous gene into the thymidine kinase gene region and/or hemagglutinin gene region of raccoon poxvirus for expression.

It is another object of the present invention to provide a method for inserting multiple genes encoding antigens of feline pathogens into the thymidine kinase gene region and/or hemagglutinin gene region of raccoon poxvirus for expression.

It is a further object of the invention to provide vaccine compositions, comprising recombinant raccoon poxvirus, for eliciting a protective immune response, to more than one feline pathogen, in felines receiving the vaccine compositions.

These and other objects of the present invention will become readily apparent from the ensuing description, embodiments and illustrations.

Brief Description of the Figures

FIG. **1** is a diagram showing the insertion of a rabies-G protein gene, operably linked to a vaccinia virus P_{11} late promoter, into a recombinant vaccinia virus recombinant plasmid carrying the FPV VP2 gene, operably linked to a vaccinia virus P_{11}, late promoter, in forming a novel plasmid termed F3S/FPV/RAB.

FIG. **2** is a representation (magnification at 250x) showing indirect immunofluorescent assay-positive plaques/cells infected with the multivalent recombinant raccoon poxvirus. Specific fluorescence, indicating expression of VP2, was concentrated in the cytoplasm of infected cells, and was also detected in syncytia cells.

FIG. **3** is a representation of labelled proteins which were precipitated by both cat anti-FPV serum and mouse anti-rabies antibody from ^{35}S-methionine-labelled RCNV/FPV infected cell lysates, in an immunoprecipitation assay for protein expression.

FIG. **4** is a graph showing viral neutralization titers against FPV in cats vaccinated (days 0, 26 and 43) with the multivalent recombinant RCNV vaccine (■), and unvaccinated controls (□) following FPV challenge.

FIG. **5** is a graph showing total white blood cell counts of all vaccinated cats (■) and control cats (□) following FPV challenge.

FIG. **6** is a diagram of the cloning of feline calicivirus (FCV) capsid protein gene and construction of a raccoon poxvirus hemagglutinin gene (HA) insertion vector carrying the FCV capsid protein gene.

FIG. **6A** is a schematic showing enzymatic amplification of the FCV capsid protein gene, and subcloning the amplified FCV fragment into a vaccinia insertion vector.

FIG. **6B** is a schematic showing construction of a fragment containing the FCV capsid gene operably linked to a promoter, and cloning the fragment into plasmid pGEM-3Z in forming a raccoon poxvirus HA insertion vector termed pGEM/HA/FCV.

FIG. **7** shows immunofluorescent antibody staining of cells infected by a recombinant raccoon poxvirus containing the FCV capsid protein gene.

FIG. **7A** shows positive plaques in a heavily infected well at lower magnification (50x).

Fig. **7B** shows positive plaques at higher magnification (500X).

Detailed Description of the Invention

The present invention provides a method for constructing recombinant RCNV which have incorporated into the viral thymidine kinase gene region and/or hemagglutinin gene region multiple exogenous genes encoding antigens of various feline pathogens. Also provided are vaccine compositions comprising the recombinant RCNV, and a method of using the vaccine compositions to elicit a protective immune response in immunized felines.

Definitions

"Expression cassette" is a term used herein for the purposes of the specification and claims to refer to a recombinant nucleic acid molecule containing multiple genes, each encoding an antigen of a feline pathogen, which are operably linked to one or more promoters such that when the expression cassette is inserted into RCNV, and upon subsequent infection of feline cells with the recombinant RCNV, the antigens encoded by their respective gene are produced by the infected feline cells.

"Feline pathogen" is a term used herein for the purposes of the specification and claims to refer to one or more microorganisms which are natural pathogens of cats, and include rabies virus, feline panleukopenia virus (FPV), feline *Chlamydia*, feline immunodeficiency virus (FIV), feline leukemia virus (FeLV), feline infectious peritonitis virus (FIPV), calicivirus, and feline herpesvirus (FHV).

The method of the present invention first comprises ligating an expression cassette into the thymidine kinase (TK) gene contained into an insertion vector such as a vaccinia virus (VV) expression vector. Because of the sequence homology between VV TK gene and the RCNV TK gene, the expression cassette within the VV

expression vector, and flanking VV TK gene sequence, is then recombined into the TK gene of raccoon poxvirus (RCNV). Alternatively, an expression cassette can be ligated into an insertion vector which has HA sequences flanking the insertion which are sufficiently homologous to promote recombination of the expression cassette into the HA gene of raccoon poxvirus. In either instance, the resultant recombinant virus can then be used as a vaccine composition in a method for immunizing felines against challenge by those feline pathogens, antigens of which are encoded by the expression cassette in the recombinant virus. For purposes of illustration and description, but not limitation, in one embodiment of the present invention the FPV VP2 gene, and the rabies virus G protein gene were each separately and operably linked to a VV late promoter in forming an expression cassette which was then recombined into raccoon poxvirus. However, the methods of the present invention provide a way to insert other exogenous genes into the raccoon poxvirus for expression upon infection into a susceptible host cell. Thus, the present invention includes compositions comprising a combination of two or more genes encoding antigens found in one or more species/ strains of feline pathogens, as will be illustrated in the following embodiments:

EXAMPLE 1

Construction of a Recombinant Insertion Plasmid

The essential features of an insertion plasmid that is useful in the method of the present invention include the following features.

(a) The plasmid sequences flanking the insertion site into which are to be inserted multiple genes, contain sequences which have sufficient homology with sequences present in the raccoon poxvirus genome to mediate

recombination. For example, a plasmid comprised of vaccinia virus sequences is used, wherein the site for insertion of multiple genes (inserted as an expression cassette) is flanked by vaccinia virus thymidine kinase gene sequences. The nucleotide sequence of the TK gene of VV is not identical to the nucleotide sequence of the TK gene of raccoon poxvirus; however, there is a sufficient degree of identity ("homology") to promote hybridization of the TK gene sequence of VV to the TK gene sequence of raccoon poxvirus and subsequent recombination. Thus, multiple genes flanked by TK gene sequence of VV are subsequently recombined into the TK gene of raccoon poxvirus. Alternatively, such flanking sequences can be part of the insert to be inserted into the plasmid.

(b) The flanking sequences must be homologous to a region of the raccoon poxvirus (into which the multiple genes are recombined) that is nonessential for the growth and propagation of the raccoon poxvirus. For example, it was found, as illustrated in Examples 2, 6 and 8-10, that both the TK gene and the hemagglutinin (HA) gene of the raccoon poxvirus genome can be used for insertion of exogenous genes by recombination. Insertion of exogenous genes into both or either of the TK gene or HA gene by recombination results in recombinant raccoon poxvirus capable of infection and replication, and can be used for expression of the recombined exogenous genes in host cells infected with the recombinant virus.

Desirable features of an insertion vector that is useful in the method of the present invention include the following features.

(a) Although it is possible that exogenous genes recombined into the raccoon poxvirus genome may be

expressed without first operably linking the genes with one or more control element elements for expression (such as a promoter) prior to recombination, operably linking the control element(s) to the multiple genes (thereby forming an expression cassette) before using the genes for insertion into the plasmid insertion vector, will likely result in higher efficiency of expression of the recombined genes. Alternatively, the sequences flanking the insertion site of the plasmid insertion vector can be engineered to contain control elements which are then operably linked to the multiple genes upon insertion.

(b) Although one promoter may be used to drive the expression of two exogenous genes to be recombined, use of two promoters in an insertion vector, each promoter operably linked to an individual exogenous gene, will provide higher efficiency of expression.

To illustrate this embodiment, first an recombinant insertion plasmid was constructed by inserting a 2,304 base pair (bp) fragment containing the FPV VP2 gene (nucleotides 1-1752 of SEQ ID NO:1), from infectious FPV genomic DNA, into a VV insertion vector. The 2,304 base pair (bp) fragment was released from the infectious clone by digestion with restriction enzyme *Hinc*II and *Sma*I. A VV insertion vector, pTKgptF3S (Baroudy et al., 1980, *J. Biol. Chem.* 255:4372-4380), was digested with *Hinc*II, treated with calf intestinal phosphatase, purified, and ligated with the FPV VP2 DNA. The ligation mixture was used to transform *Escherichia coli* DH5 alpha. The transformed *E. coli* cells were plated on Lauria broth (LB) agar plates with 50 µg/ml ampicillin and incubated overnight at 37°C. The colonies grown on the agar plates were screened by *in situ* colony hybridization using a ^{32}P-labeled FPV VP2

probe. The correct orientation and the proper open reading frame were further confirmed by restriction enzyme mapping and DNA sequencing. A plasmid, termed F3S/FPV, contained the VP2 gene in the correct orientation in relation to the operably linked VV P_{11} promoter.

A DNA fragment containing the rabies-G gene (SEQ ID NO:2) operably linked to a VV P_{11} promoter (SEQ ID NO:3) was purified from a RCNV rabies-G recombinant virus described previously (Ngichabe, C.K., 1992, Ph.D. Thesis, Cornell University, Ithaca, New York). The RCNV rabies-G recombinant virus was grown in CV-1 cell monolayers. When the cytopathic effect (CPE) reached about 80% of the cell monolayer, the cells were scraped off, washed in PBS with 100 μM $MgSO_4$ (PBS-M) and resuspended in 1,200 μl of PBS-M. The virus was released from the infected cells by denaturation solution (0.5% Triton, 45 mM mercaptoethanol and 20 mM EDTA). The cell lysate was centrifuged at 300 x g to separate the chromosomal DNA and debris, then the viruses were pelleted from the supernatant by centrifugation at top speed of the microcentrifuge for 10 minutes, and suspended in 100 μl of Tris-EDTA buffer (TE), pH 7.5. After treatment with 150 μg/ml proteinase K, 200 mM NaCl, and 45 mM mercaptoethanol. The mixture was mixed gently and incubated for 2 hours in a 50°C waterbath. After extraction twice with equal volume of Tris buffered phenol:chloroform:isoamyl alcohol and four times with water saturated ether and ethanol precipitation. The DNA was pelleted by centrifugation at top speed at 4°C in a microcentrifuge, washed in 70% cold alcohol, resuspended in 100 μl of water and then stored at -70°C for use.

A DNA fragment containing the gene encoding rabies-G was isolated, and cloned into F3S/FPV, as follows (See also Fig. 1). A 1.75 Kb fragment containing the rabies-G gene operably linked to a P_{11} late promoter was amplified by polymerase chain reaction (PCR) from purified RCNV rabies-G recombinant virus DNA using a forward primer (SEQ ID NO:4) and a reverse primer (SEQ ID NO:5). After separation of the amplified DNA on 1% agarose gel, the amplified DNA was digested by *BamH*I and *Kpn*I which were designed on both ends of the PCR primers. The reverse primer had an artificial restriction enzyme digestion site of *BamH*I which was aimed at the tail *BamH*I site of the FPV VP2 gene (Fig.1). The forward primer carried an artificial restriction enzyme digestion site of *Kpn*I which was aimed at the *Kpn*I site in the gpt fragment of the F3S/FPV plasmid (Fig. 1). The DNA bands were extracted, reseparated on the 1% agarose gel and recovered by a DNA purification kit. The DNA fragments, comprising linearized F3S/FPV and the amplified DNA which had been restricted, were ligated together and the ligation mixture was used to transform *E. coli* DH5α. The transformed *E. coli* cells were plated on the LB agar plate containing 50 μg/ml ampicillin, and incubated at 37°C overnight. The amp-resistant selected bacterial colonies were screened by colony hybridization using both FPV VP2 and rabies G genes as probes (SEQ ID NO:1 and SEQ ID NO:2, respectively). The colonies positive by hybridization were then selected and cultured in the LB medium for preparation of plasmid DNA. A plasmid, termed F3S/FPV/RAB, was formed which contained rabies-G gene operably linked to a P_{11} and the FPV VP2 gene operably linked to a VV P_{11} promoter (Fig. 1). The purified plasmid DNA was further confirmed by DNA

sequencing. Three primers were used to sequence both FPV VP2 and rabies G genes (SEQ ID NOs: 4, 5, and 6). A primer (SEQ ID NO:6) was used to sequence the promoter, the junction of promoter, and VP2 coding sequence. The two PCR primers (SEQ ID NO:4 and SEQ ID NO:5) were used to sequence the inserted PCR fragment. All of the sequenced DNAs were compared with the original published sequences. Note that in F3S/FPV/RAB, the respective exogenous genes, operably linked to their respective promoter, are in opposite orientation (in the direction of transcription) relative to each other. Each inserted gene had its own promoter, start codon and stop codon for authentic protein expression. Thus, both inserted genes were not mutated in this multivalent plasmid.

EXAMPLE 2

Construction of a Recombinant Raccoon Poxvirus

The essential features of a potential viral vaccine vector for use in the immunization of felines according to the present invention include the following features.

(a) The viral vector need be safe and suitable as a vaccine in cats. For example, the viral vector must infect and replicate in cats without causing significant pathology in immunized cats. Also, the viral vector must be capable of expressing genes recombined into it, upon infecting feline cells.

(b) The viral vector must be able to tolerate large foreign insertions (multiple genes inserted by recombination) into either or both of the thymidine kinase gene and the hemagglutinin gene without significantly and negatively affecting the ability of the recombinant virus to infect and replicate in susceptible host cells.

Raccoon poxvirus (RCNV) was found to be safe and suitable as a viral monovalent vaccine vector useful for immunizing cats (Ngichabe, C.K., 1992, *supra*). However, it was not known at the time of the invention whether raccoon poxvirus could tolerate large foreign insertions, as required when recombining multiple genes/ expression cassettes (versus a single gene), into the thymidine kinase gene or its hemagglutinin gene of the raccoon poxvirus genome without the virus losing its ability to infect and replicate in susceptible host cells. Further, it was not known at the time of the invention whether there were any constraints relative to the orientation of insertion of multiple genes with respect to the direction of transcription. Extrapolating the results of studies with VV vectors, relative to the capacity of the viral genome to tolerate insertions and relative to orientation, to RCNV would be presumptuous because the DNA sequences between RCNV and VV are significantly different.

The multivalent RCNV-based recombinant FPV and rabies virus was generated by homologous recombination of the flanked vaccinia virus TK gene sequence connected to both ends of the two insertions (VP2 and rabies-G) with the TK fragment of RCNV. Purified plasmid DNA containing both FPV VP2 and rabies-G genes, with each gene operably linked to a P_{11} promoter, was transfected by calcium phosphate precipitation into CV-1 cell monolayers which were also infected with raccoon poxvirus. When the plaques reached about 60% confluence, the transfection mixture was harvested and freezethawed three times. The recombinant RCNV virus was selected by plaque purification in the presence of 50 µg/ml 5-bromo-2'deoxyuridine (BUDR), and dot blot hybridization using ^{32}P-labelled FPV VP2 and rabies-G

gene probes (SEQ ID NO:1 and SEQ ID NO:2, respectively). The positive plaque was further plaque purified three times in rat-2 TK cells with the presence of BUDR and then grown in CV-1 cells without BUDR. The cells were harvested 1 to 2 days later when maximum CPE was observed. The cell suspension was frozen and thawed three times and sonicated before use as vaccine. If desired, the recombinant RCNV can be pelleted by gradient centrifugation, resuspended, and titrated on Rat-2 cell monolayers, to determine the pfus.

EXAMPLE 3

Molecular analysis of the multivalent recombinant raccoon poxvirus

In this embodiment, the multivalent recombinant raccoon poxvirus was analyzed for the presence of the VP2 gene and rabies-G gene. The multivalent recombinant raccoon poxvirus was purified by plaque-purification and selected by dot-blot hybridization using ^{32}P-labelled VP2 and rabies-G gene probes (SEQ ID NO:1 and SEQ ID NO:2, respectively), as described in Example 2. Further, using DNA isolated from the purified multivalent recombinant raccoon poxvirus, an internal sequence of each of the FPV VP2 and rabies-G genes were enzymatically amplified. Two bands were observed when the enzymatically amplified DNA was run on a 1% agarose gel. The bands were the expected sizes of the rabies-G gene internal sequence, and the FPV VP2 internal sequence. The gel was also probed with both the radiolabelled VP2 and rabies G gene probes after Southern transfer; and the results verify the presence of the VP2 and rabies-G genes in the multivalent recombinant raccoon poxvirus.

EXAMPLE 4

Expression of VP2 and rabies-G proteins in the cells infected with multivalent recombinant raccoon poxvirus

In this embodiment, the multivalent recombinant raccoon poxvirus was analyzed for expression of the recombined multiple genes.

4.1 Indirect Immunofluorescent antibody assay (IFA)

An IFA was performed to detect expression of VP2 and rabies-G by cells infected with the multivalent recombinant raccoon poxvirus. Briefly, the CV-1 or CrFK (Crandell feline kidney) cell monolayers, grown in 8-well chamber slides, were infected with the multivalent recombinant virus at one multiplicity of infection. After fixation in cold acetone for 10 minutes, the cell monolayer was washed in PBS and immersed with 1:10 diluted normal cat serum (from specific-pathogen-free cats; "SPF" cats) for 10 minutes. The slides were then incubated with cat anti-FPV polyclonal antibody diluted 1:200 or mouse anti-rabies-G monoclonal antibody for one hour. The slides were thoroughly washed in PBS and incubated with fluorescein-labelled rabbit anti-mouse antibody for 30 minutes. After washing in PBS, the slides were sealed by glycerol and examined by immunofluorescence microscopy.

Immunofluorescence was detected when either cat anti-FPV serum or mouse anti-rabies G monoclonal antibody was reacted with cells infected with multivalent recombinant virus. infected cells or plaques. Fig. 2 shows the IFA-positive plaques/cells infected with the multivalent recombinant raccoon poxvirus. Bright green fluorescence was mostly concentrated on the cytoplasm of the infected cells. Also, IFA detected positive particles in the center of

some positive plaques. No difference in strength or patterns of fluorescence was observed when IFA was performed with FPV polyclonal antibody versus with rabies monoclonal antibody.

4.2 Immunoprecipitation assay

An immunoprecipitation assay was performed to detect expression of VP2 and rabies-G by cells infected with the multivalent recombinant raccoon poxvirus. ^{35}S-methionine-labelled cell lysate, from CV-1 cells infected with the multivalent recombinant raccoon poxvirus, was immunoprecipitated with cat anti-FPV antiserum and mouse anti-rabies-G monoclonal antibody. Briefly, the CV-1 cell monolayers were infected with the multivalent recombinant raccoon poxvirus, and then were pulse-labelled with ^{35}S-methionine for 12 hours. The infected cells were lysed in lysis buffer, and all solubilized cellular proteins were separated from the cell debris by centrifugation. The clarified protein suspension was precipitated with anti-FPV antibody or anti-rabies-G monoclonal antibody. The precipitated proteins were loaded onto an 8% to 25% gradient polyacrylamide gel and electrophoresed. The mini-gel was directly fixed, enhanced, and finally neutralized before exposure to X-ray film.

Precipitated proteins, precipitated by both cat anti-FPV serum and mouse anti-rabies antibody from the ^{35}S-methionine-labelled RCNV/FPV infected cell lysate, are indicated in Fig. 3. The expression of three proteins was observed by the immunoprecipitation assay: FPV VP2 protein of 67 kilodalton (Kd) (Fig. 3, lane 1); and rabies-G1 of 64 Kd, and rabies-G2 of 62 Kd (lane 2). The two rabies bands were very clear if the exposure time was shorter. Lane 4, containing a control

cell lysate infected with wild type RCNV, did not show similar bands in the immunoprecipitation assay.

EXAMPLE 5

Vaccine studies with multivalent recombinant raccoon poxvirus

In this embodiment, the multivalent recombinant raccoon poxvirus was used to immunize cats to induce an immune response against the feline pathogen antigens expressed by cells infected the multivalent recombinant raccoon poxvirus (RCNV/FPV/RAB-G).

5.1 Preparation of vaccine stock

The vaccine stock for the RCNV/FPV/RAB-G construct was prepared using a previously described method for preparing raccoon poxvirus recombinants for use in vaccines (Esposito et al., 1988, *Virology* 165:313-316). CrFK cell monolayers, grown in 150 cm^2 tissue culture flasks, were infected with plaque-purified RCNV/FPV/RAB-G at multiple of infectivity = 0.5. The multivalent recombinant virus was allowed to adsorb at 37°C for 2 hours. Supplemented tissue culture medium was added, and the cultures were incubated 24 hours at 37°C. When the confluent CPE was reached, the cells were scraped, concentrated by polyethylene glycol (PEG 8000) and pelleted at 16,000 x g for 30 minutes. The pellet was suspended in 1.0 ml of Tris-Cl EDTA (TE), pH 7.6, for every 100 ml of starting culture fluid. Sodium diatrizoate composite gradients were used for layering the suspended mixture. The gradient contained 1 ml of 50% diatrizoate, 25 ml of 25-50% diatrizoate, and 3 ml of Dextran-10. After centrifugation at 125,000 x g for 16 hours at 4°C, the viral band was collected, diluted four-fold in TE buffer, and centrifuged through a 30%

sucrose cushion. The viral pellet was then resuspended, sonicated, titrated on Rat-2 cells and diluted in 50% v/v in sterile glycerol and TE buffer. The vaccine mixture was finally stored at -20°C for later use.

5.2 Design of vaccination and challenge studies

The animals used in the vaccination and challenge studies were five- to 6-month-old specific-pathogen-free cats (SPF cat colony) that were kept in regulatory-approved biohazard isolation units. Group A consisted of 9 cats for vaccination. Group B had 2 cats that were unvaccinated controls. Group A was vaccinated subcutaneously three times on days 0, 26, and 34 with recombinant RCNV carrying both FPV VP2 and rabies G genes (RCNV/FPV/RAB-G). Each cat received a dose of 1 X 10^7 PFU virus each time. The control cats received phosphate buffered saline (PBS) at the same time as group A was vaccinated. Sixteen days following the third vaccination, all cats were challenged orally with $10^{4.5}$ $TCID_{50}$ of FPV challenge virus (National Veterinary Service, Ames, Iowa.) strictly following the USDA challenge procedure.

5.3 Vaccination and challenge results

5.3.1 Virus neutralization antibody titer

All cats in the vaccination and challenge studies were checked daily for clinical signs. Blood was collected for total white blood cell counts at days 0, 3, 5, 7 and 10 after challenge, and the sera were collected on days 26, 43, 50 and 57 after vaccination. All sera were aliquotted into two vials for both FPV antibody and rabies antibody tests. The viral neutralization antibody assay for antibodies against rabies virus was done by the Rabies Virus Laboratory,

New York State Department of Health, Albany, New York. The viral neutralization test for rabies virus was done by the rapid immunofluorescent focus inhibition test. The rabies neutralization antibody titers were interpreted as international unit (IU) per ml (1 IU = 1:32 virus neutralization titer).

The serum from each cat was also assayed by viral neutralization antibody assays for anti-FPV antibody titer. Briefly, CrFK cells were seeded on 8-chamber slides at $1x10^5$ cells/ml, and incubated at 37°C for one hour. Two-fold serial dilutions of all collected serum samples were mixed with an equal volume of 32-100 $TCID_{50}$/0.1 ml of FPV, and were incubated at 37°C for another hour. The mixtures were then added to individual chambers seeded with CrFK cells and incubated at 37°C in a 5% CO_2 atmosphere for 3 to 4 days. The infected cell monolayers were fixed with methyl alcohol, stained with May Grüwald-Giemsa stain and examined under light microscope for FPV intranuclear inclusions. The highest dilution of serum at which no detectable FPV inclusion bodies were identified was interpreted as the virus neutralization titer.

For the FPV virus neutralization assay, the eight cats vaccinated with the multivalent recombinant raccoon poxvirus (RCNV/FPV/RAB-G) achieved a mean virus neutralization titer of 1:1,000 at day 27 (Fig. 4). The virus neutralization titer reached 1:7,000 at day 43, and over 1:10,000 by day 50 (Fig. 4). All three vaccinations boosted the FPV virus neutralization titer, which was not affected by the FPV challenge in vaccinated cats. However, the unvaccinated cats (Group B) obtained virus neutralization titer only after day 50 when the FPV challenge was given. The virus neutralization titer in Group B cats, following

challenge with FPV, was as high as 1:5,000 at day 60 (Fig. 4).

For the rabies virus neutralization assay (Table 1), the virus neutralization titer was as high as 4 to 16 international unit (IU) at day 43; and up to 8 to 16 IU at day 50 after vaccination. The virus neutralization titers of two vaccinated cats were ≥ 8 international units (IU) at day 26; and the titers of 4 vaccinated cats rose to a ≥ 16 IU by day 43. All of the vaccinated cats obtained a virus neutralization titer over 8 IU at day 50, and four out of 8 vaccinated cats obtained a virus neutralization (VN) titer over 16 IU by day 57 after vaccination. However, the control unvaccinated cats were negative throughout the experiment (Table 1)

Table 1

		RABIES VN TITERS (IU)				
	Date Cat #	0	26	43	50	57
Unvaccinated cats	A981	<0.063	<0.063	<0.063	<0.063	<0.063
	A932	<0.125	<0.125	<0.125	<0.125	<0.125
Vaccin-ated Cats	A983	<0.063	1	4	>=8	>=8
	A954	<0.063	2	>=8	>=8	>=8
	A962	<0.063	2	4	>=8	>=8
	A931	<0.063	>=8	>=8	>=8	>=8
	A964	<0.125	4	>=16	>=16	>=16
	A982	<0.125	1	>=16	>=16	>=16
	A955	<0.125	1	>=16	>=16	>=16
	A963	<0.125	8	>=16	>=16	>=16

(1 IU = 1:32 VN titer)

5.3.2 Leukocyte counts

As described above, blood was collected for total white blood cell counts at days 0, 3, 5, 7 and 10 after challenge. The total white blood cells and differential counts were determined using an automated hematology analyzer. The clinically critical indication of the FPV infection is the change in white blood cell (WBC) counts during the first 10 days after FPV infection. As shown in Fig. 5, total WBC counts of all vaccinated cats were stable in the clinically no=al range throughout the experiment (■). The mean WBC counts of the unvaccinated cats were typical of FPV infection. The mean counts were as low as 2.3 thousand/microliter at day 5 after the FPV challenge (□) and the cats were fully recovered at day 20 when the experiment was ended. This critical indicator of an immune response to FPV demonstrated strong protection of vaccinated cats from subsequent FPV challenge.

Taken together, the vaccination studies indicate that cats vaccinated with the multivalent recombinant raccoon poxvirus were fully protected from FPV challenge, and provoked strong humoral immune responses which may be sufficient to protect the cats from rabies infection. Due to the regulatory restriction of rabies challenge, the efficiency of rabies protection was based on the serum virus neutralization antibody titers following vaccination. However, previous studies have showed that a virus neutralization titer of 0.5 IU is sufficient for protection against rabies virus challenge (Barth et al., 1988, *Vaccine* 6:369-377; Ngichabe, 1992, *supra*). Thus, the virus neutralization titers of cats vaccinated with the multivalent recombinant raccoon poxvirus vaccine were over 32 fold higher than the recommended

satisfactory titers. Based on the data, it is believed cats vaccinated with such a multivalent recombinant raccoon poxvirus would be protected from subsequent rabies virus challenge.

EXAMPLE 6

6.1 Feline calicivirus as a feline pathogen antigen in a multivalent recombinant raccoon poxvirus vaccine

Feline calicivirus (FCV) is an important pathogen of cats causing serious feline diseases including acute oral ulceration, mild upper respiratory diseases and severe lower respiratory diseases, and febrile lameness syndrome. In infected cats, FCV is shed in ocular, nasal, and pharyngeal secretions. Cats that have recovered from FCV infection may also become persistent shedders of the virus. The capsid protein of FCV in FCV-infected cells is a 76 Kd protein. Neutralizing epitopes exist on the capsid protein. The nucleotide sequence of the FCV capsid protein was reported recently (Seal et al., 1993, *J. Gen.* Virol. 74:2519-2524; Seal et al., 1995, *Virus Genes* 9:183-187). A cDNA clone of the capsid protein gene (SEQ ID NO:7), from highly virulent strain FCV 255, was used in the construction of a multivalent vaccine as follows.

Using the methods of the present invention, as illustrated in Examples 1-3, an expression cassette can be constructed to include any combination of two or more genes, wherein the genes encode the feline calicivirus (FCV) capsid protein (SEQ ID NO:7), FPV VP2 (SEQ ID NO:1), or rabies-G (SEQ ID NO:2). The expression cassette may then be inserted into an insertion vector, and subsequently recombined into the TK gene of raccoon poxvirus, thereby forming a multivalent recombinant raccoon poxvirus.

Alternatively, genes encoding two or more feline pathogen antigens can be recombined into the hemagglutinin (HA) gene of raccoon poxvirus in forming a multivalent recombinant raccoon poxvirus. In another embodiment, using the methods according to the present invention, both the HA gene and the TK gene can be used as sites into which at least one gene encoding a feline pathogen antigen is recombined into each site (recited in the claims as "a combination thereof"). The vaccinia virus HA gene has been used for construction of vaccinia HA insertion vectors (Shida, 1989, *Subcell. Biochem.* 15:405-440; Shida et al., 1983, Cell 33:423-434). The HA sequence is not essential for virus reproduction or infectivity in cell culture, but it affects the way that the virus is disseminated. The HA glycoprotein, synthesized by rough endoplasmic reticulum in the plasma membrane, gives both hemagglutination and hemadsorption. Thus, using hemagglutination and hemadsorption assays, mutations of the HA gene allow screening and selection of the HA phenotype.

Analysis of the HA genes of both RCNV and vaccinia virus showed homology of 69% in DNA sequences and 53% in amino acids of the HA between RCNV and VV (Cavallaro et al., 1992, *Virology* 190:434-439). Thus, the low HA homology between the two viruses excludes the possibility of, and would teach against one skilled in the art to attempt, using vaccinia HA insertion vectors for homologous recombination into the RCNV HA gene.

6.2 Construction of a Recombinant HA Insertion Plasmid

The essential features of an HA insertion plasmid that is useful in the method of the present invention include the following features.

(a) The plasmid sequences flanking the insertion site into which are to be inserted multiple genes, contain sequences which have sufficient homology with sequences present in the raccoon poxvirus genome to mediate recombination. Thus, to construct such a HA insertion vector, raccoon poxvirus HA sequence was used as the flanking sequences to position a vaccinia virus promoter and the insert containing one or more genes that is expected to be expressed. Although the HA flanking sequences can be synthesized to vary slightly from the HA sequence found in RCNV, there must be a sufficient degree of identity ("homology") to promote hybridization of the HA flanking sequence to the HA gene sequence of raccoon poxvirus and subsequent recombination. Alternatively, such flanking sequences can be part of the insert to be inserted into the plasmid.

(b) The flanking sequences must be homologous to a region of the raccoon poxvirus (into which the multiple genes are recombined) that is nonessential for the growth and propagation of the raccoon poxvirus. As illustrated in this Example, the hemagglutinin (HA) gene of the raccoon poxvirus genome can be used for insertion of exogenous genes by recombination. Insertion of exogenous genes into the HA gene by recombination results in recombinant raccoon poxvirus capable of infection and replication, and can be used for expression of the recombined exogenous genes in host cells infected with the recombinant virus.

Desirable features of an insertion plasmid that is useful in the method of the present invention include the following features.

(a) Although it is possible that exogenous genes recombined into the raccoon poxvirus genome may be expressed without first operably linking the genes with

one or more control element elements for expression (such as a promoter) prior to recombination, operably linking the control element(s) to the multiple genes (thereby forming an expression cassette) before using the genes for insertion into the plasmid insertion vector, will likely result in higher efficiency of expression of the recombined genes. Alternatively, the sequences flanking the insertion site of the plasmid insertion vector can be engineered to contain the control elements which are then operably linked to the multiple genes upon insertion.

(b) Although one promoter may be used to drive the expression of two exogenous genes to be recombined, use of two promoters in an insertion vector, each promoter operably linked to an individual exogenous gene, will provide higher efficiency of expression.

Thus, in this embodiment, RCNV HA sequence was used as flanking sequences to position a promoter operably linked to the foreign gene insert that is to be expressed. To illustrate this embodiment, a 2381 bps cDNA fragment amplified from the pSV.SPORT1/FCV cDNA clone (Fig. 5) was flanked with an *Xho*I site at the 5'-start codon end and *Kpn*I at the 3' end. A vaccinia TK insertion plasmid, pMJ601, was digested with *Sal*I and *Kpn*I. The FCV fragment, containing the capsid protein gene, was ligated into pMJ601 to form a plasmid, termed pMJ601/FCV. By such insertion, the FCV capsid protein gene was operably linked to a vaccinia late promoter. The plasmid DNA was sequenced to confirm the accuracy of the cloned fragment, and the open reading frame orientation.

A RCNV HA fragment of 1,262 base pairs (bp) was divided into two parts from which a HA left arm (HAL) and a right arm (HAR) were prepared by enzymatic

amplification using the polymerase chain reaction (PCR). The HA left arm of 582 bps (SEQ ID NO:8) and the HAR of 447 bps (SEQ ID NO:9) were amplified using primers P1, P2, P3 and P4 (Fig. 6). The middle portion of the HA gene (between HAL and HAR) was deleted after forming the two end fragments. The amplified products of HAL, HAR, and of the capsid protein gene operably linked to a promoter (P/FCVCP), were joined and amplified by recombinant PCR. The full sequence, which contained HAL-P/FCVCP-HAR of 3,353 bp was prepared from two recombinant fragments. Conditions typically used for enzymatic amplification, include a denaturation step at 95°C for 1 minute, an annealing step at 60°C for 1 minute, an extension at 72°C for 3 minutes, an extra extension at 72°C for 7 minutes, and a soaking step at 4°C for analysis. For FCV capsid protein gene amplification, an annealing temperature at 68°C was applied and other temperatures were the same as above. Enzymatically amplified fragments of HAL and P/FCVCP were joined by recombinant PCR using the 20 bps overhang sequence of a primer, P3 (SEQ ID NO:10). About 10 to 50 ng of purified DNA fragments of HAL and P/FCVCP were mixed with a thermostable DNA polymerase without primers. The first three cycles of PCR were designed for ligation of the two fragments in the presence of the thermostable DNA polymerase. The protocol of the thermocycle steps was as follows: denature at 94°C for 1 minute, anneal at 40°C for 2 minutes at a ramp rate of 6 seconds/per degree and extension at 72°C for 3 minutes at ramp rate of 2 seconds/per degree. Primers P1 (SEQ ID NO:11) and F2 (SEQ ID NO:12) were added to the reaction tube and a regular PCR was performed for 30 cycles with denaturation at 94°C for 1 minute, annealing at 55°C for 1 minute and extension at 72°C for 2 minutes

(See also, Fig. 6A). A final incubation at 72°C for 7 minutes was used for the extension of all amplified fragments. Using the same protocol and conditions, amplified fragments of P/FCVCP and HAR were ligated by primers P5 (SEQ ID NO:13) and P2 (SEQ ID NO:14) (See also, Fig. 6B). All of the amplified products were purified from 1% agarose gel by a DNA purification kit.

The low annealing temperature (40°C) and ramp temperature during the turnover of each thermocycle step greatly facilitated the process of recombination. After two to three cycles without primer, the ligated fragment was amplified by PCR. The resultant recombinant product, HAL-P/FCVCP-HAR, was ultimately subcloned into plasmid pGEM-3Z to form a novel RCNV HA insertion plasmid carrying FCV, termed pGEM/FCV. The total length of the plasmid was 6175 bps which included 3353 bps of HAL-P/FCVCP-HAR and 2726 bps of pGEM (Fig. 6B).

<u>6.3 Construction of a Recombinant Raccoon Poxvirus</u>

Homologous recombination between the RCNV HA sequences of pGEM/FCV and between the HA sequences of wild type RCNV was performed with procedures outlined in Example 2, and as described previously (See for example, Mackett et al., *J. Virol.* 49:857-864, herein incorporated by reference). Briefly, the purified HA insertion vector, pGEM/FCV, was precipitated onto a CV-1 cell monolayer infected with wild type RCNV by calcium phosphate transfection. After 48 hours of the transfection, the cell monolayer was frozen and thawed three times. The virus mixture was plated at different dilutions onto the CV-1 monolayer and grown in the 8-well chamber slide. When CPE appeared, the cells were fixed and stained with cat anti-FCV serum.

6.4 Expression of FCV capsid protein in cells infected with a Recombinant Raccoon Poxvirus

Indirect immunofluorescent antibody assays were performed to detect FCV-specific fluorescence in the cytoplasm and plasma membrane in infected cells and recombinant raccoon poxvirus-formed plaques in cell culture. CV-1 cell monolayers were infected with the recombinant RCNV or wild type RCNV and incubated for 18 to 24 hours at 37°C. The infected cell monolayers were fixed in cold acetone, incubated first with 1:10 diluted normal cat serum for 10 minutes, and then with 1:100 diluted cat anti-FCV antiserum for one hour at room temperature. The monolayer was finally stained in 1:100 diluted fluorescein labelled rabbit anti-cat IgG for 30 minutes and examined by fluorescent microscopy. The immunofluorescent antibody assay showed that positive viral plaque and/or positive infected cells were detectable by specific FCV antibody. Fig. 7A shows two separate positive plaques in a heavily infected well at lower magnification. Fig. 7B shows recombinant virus-induced plaques at higher magnification. The cytoplasm of the infected cells and the syncytia cells were heavily stained with intensive fluorescence. Even though the fluorescent particles were more likely concentrated around the nucleus, the positive particles were distributed all over the cytoplasm and plasma membrane. All of the wild type RCNV-infected cells were negative.

6.4 Vaccines comprising Recombinant Raccoon Poxvirus containing FCV capsid protein gene

Using the methods according to Example 5, vaccine stocks of recombinant raccoon poxvirus

containing FCV capsid protein gene can be prepared, and administered in a vaccination process to felines.

EXAMPLE 7

Alternatives in feline pathogen antigen expression

The selection of a promoter may depend on the feline pathogen antigen to be expressed. Promoters vary in strength, i.e. ability to facilitate transcription. Generally, for the purpose of expressing a cloned gene, it is desirable to use a strong promoter in order to obtain a high level of transcription of the gene and expression into gene product. For example, viral promoters known in the art, which may be used in a multivalent recombinant raccoon poxvirus, from which a high level of transcription has been observed in infected mammalian cells include the SV40 early promoter, CMV promoters, various vaccinia promoters, adenovirus major late promoter, and the like, may be used to provide transcription of the inserted DNA sequence encoding feline pathogen antigens.

To improve the efficiency of expression of feline pathogen antigens, it is preferable that each gene encoding an antigen is operably linked to a promoter. However, for some promoters, such as the vaccinia virus P_{11} promoter, apparently two genes with the same kind of promoter can not be oriented in the same direction in the recombinant raccoon poxvirus. Thus, a gene to be expressed may have to be inserted in a transcription orientation which is opposite to the transcription orientation of an adjacent gene to be expressed.

Other control elements for efficient gene transcription or message translation include enhancers, and regulatory signals. Enhancer sequences are DNA

elements that appear to increase transcriptional efficiency in a manner relatively independent of their position and orientation with respect to a nearby gene. Thus, an enhancer may be placed either upstream or downstream from the inserted DNA sequences encoding feline pathogen antigens to increase transcriptional efficiency.

Further, genetic engineering techniques can be used to reduce a gene to a gene fragment that encodes only a portion of the feline pathogen antigen; but a portion that acts as an immune response-inducing epitope. For example, from the sequences of the various genes feline pathogen, it can be determined which restriction enzyme or combination of restriction enzymes may be used to generate sequences encoding immunogenic peptides. Restriction enzyme selection may be done so as not to destroy the immunopotency of the resultant peptide. Antigenic sites of a protein may vary in size but can consist of from about 7 to about 14 amino acids. Thus, a protein the size of most feline pathogen antigens may contain many discrete antigenic sites; therefore, many partial gene sequences could encode antigenic epitopes. Consequently, using the nucleotide sequence of the particular gene as a guide, restriction enzyme combinations may be used to generate DNA sequences, which when inserted into a multivalent recombinant raccoon poxvirus, are capable of directing the production of peptides comprising one or more antigenic epitopes.

Modification of the feline pathogen antigens or peptides, such as by deletion and substitution of amino acids (and including extensions and additions to amino acids) and in other ways, may be made so as to not substantially detract from the immunological properties

of the antigen or peptide. In particular, the amino acid sequence of the antigen or peptide, may be altered by replacing one or more amino acids with functionally equivalent amino acids resulting in an alteration which is silent in terms of an observed difference in the physicochemical behavior of the antigen or peptide. Functionally equivalent amino acids are known in the art as amino acids which are related and/or have similar polarity or charge. Thus, an amino acid sequence which is substantially that of the amino acid sequences depicted in the Sequence Listing herein, refers to an amino acid sequence that contains substitutions with functionally equivalent amino acids without changing the primary biological function of antigen, or peptide.

Also, it will be appreciated by those skilled in the art, that because of third base degeneracy, almost every amino acid is represented by more than one triplet codon in a coding nucleotide sequence. Thus, a gene encoding a feline pathogen antigen as disclosed herein, may be modified slightly in nucleotide sequence, and yet still encode its respective gene product of the same amino acid sequence. Thus, insertion of such modified genes into a multivalent recombinant raccoon poxvirus is within the scope of the present invention.

EXAMPLE 8

<u>Feline infectious peritonitis virus as a feline pathogen antigen in a multivalent recombinant raccoon poxvirus vaccine</u>

Feline infectious peritonitis virus (FIPV) is a coronavirus which causes a highly fatal disease in infected cats. It has been shown that in coronavirus infections, antibodies may be developed against the membrane glycoprotein (M) and the nucleocapsid protein

(N) which can inhibit virus replication. The nucleotide sequences of the FIPV M (SEQ ID NO:17) and N (SEQ ID NO:18) genes, and their deduced amino acid sequences, have been described previously (Vennema et al., 1991, *Virology* 181:327-335; the disclosure of which is herein incorporated by reference).

Using the methods of the present invention, as illustrated in Examples 1-3, and 7, an expression cassette can be constructed to include any combination of two or more genes, wherein one of the genes encodes either the FIPV M or N protein. Other exogenous genes that can be included in such an expression cassette include genes that encode FCV capsid protein, FPV VP2, or rabies-G. The expression cassette may then be inserted into an insertion vector, and subsequently recombined into the TK gene of raccoon poxvirus, thereby forming a multivalent recombinant raccoon poxvirus.

Alternatively, using the methods of the present invention, as illustrated in Examples 1-4, 6, and 7, an expression cassette can be constructed to include any combination of two or more genes, wherein one of the genes encodes either the FIPV M or N protein. Other exogenous genes that can be included in such an expression cassette include genes that encode the FCV capsid protein, FPV VP2, or rabies-G. The expression cassette may then be inserted into an insertion vector, and subsequently recombined into the HA gene of raccoon poxvirus, thereby forming a multivalent recombinant raccoon poxvirus.

In another embodiment, using the methods according to the present invention, both the HA gene and the TK gene can be used as sites into which exogenous genes encoding feline pathogen antigens are recombined into raccoon poxvirus. Combinations of such exogenous

genes include genes which encode FIPV M, FIPV N, FCV capsid protein, FPV VP2, and rabies-G. With all of these embodiments, using the methods according to Example 5 and other methods known in the art, vaccine stocks of recombinant raccoon poxvirus containing exogenous genes encoding feline pathogen antigens can be prepared, and administered in a vaccination process to felines.

EXAMPLE 9

<u>Feline immunodeficiency virus as a feline pathogen antigen in a multivalent recombinant raccoon poxvirus vaccine</u>

Feline immunodeficiency virus (FIV) is a retrovirus which causes a persistent generalized lymphadenopathy, recurrent fevers, anorexia, and weight loss in infected cats. Often chronic secondary infections are present which are caused by other feline pathogens. A comprehensive review of FIV biology, infection, and immune responses thereto, has been recently published (Bendinelli et al., 1995, *Clin. Microbiol. Reviews* 8:87-112; the disclosure of which is hereby incorporated by reference). In vaccine studies, cats receiving immunizations with either inactivated whole-infected cell or cell-free feline immunodeficiency virus (FIV) vaccines were protected against subsequent FIV challenge; wherein protection appeared to correlate with antiviral envelope antibody titers (Yamamoto et al., 1993, *J. Virol.* 67:601-605). The gene encoding FIV Gag protein (approximately FIV nucleotide sequence base 600 to base 2,000; Bendinelli et al., *supra),* or the gene the FIV Env protein (approximately FIV nucleotide sequence base 6,250 to base 8,850; Bendinelli et al., *supra)* may be used to induce neutralizing antibodies which may inhibit virus infection and/or replication.

Using the methods of the present invention, as illustrated in Examples 1-4, and 7, an expression cassette can be constructed to include any combination of two or more genes, wherein one of the genes encodes either the FIV Gag or Env protein. Other exogenous genes that can be included in such an expression cassette include genes that encode FIPV M, FIPV N, FCV capsid protein, FPV VP2, or rabies-G. The expression cassette may then be inserted into an insertion vector, and subsequently recombined into the TK gene of raccoon poxvirus, thereby forming a multivalent recombinant raccoon poxvirus.

Alternatively, using the methods of the present invention, as illustrated in Examples 1-4, 6, and 7, an expression cassette can be constructed to include any combination of two or more genes, wherein one of the genes encodes either FIV Gag or FIV Env. Other exogenous genes that can be included in such an expression cassette include genes that encode FIPV M, FIPV N, FCV capsid protein, FPV VP2, or rabies-G. The expression cassette may then be inserted into an insertion vector, and subsequently recombined into the HA gene of raccoon poxvirus, thereby forming a multivalent recombinant raccoon poxvirus.

In another embodiment, using the methods according to the present invention, both the HA gene and the TK gene can be used as sites into which exogenous genes encoding feline pathogen antigens are recombined into raccoon poxvirus. Combinations of such exogenous genes include genes which encode FIV Gag, FIV Env, FIPV M, FIPV N, FCV capsid protein, FPV VP2, and rabies-G. With all of these embodiments, using the methods according to Example 5 and other methods known in the art, vaccine stocks of recombinant raccoon poxvirus

containing exogenous genes encoding feline pathogen antigens can be prepared, and administered in a vaccination process to felines.

EXAMPLE 10

Feline leukemia virus as a feline pathogen antigen in a multivalent recombinant raccoon poxvirus vaccine

Feline leukemia virus (FeLV) is a oncornavirus which causes leukemia and related symptoms in infected cats. FeLV env gene expression via a canarypox virus-based vector, was used as the basis for a vaccine in cats (Tartaglia et al., 1993, *J. Virol*.67:2370-2375). The nucleotide sequence of the FeLV env gene (SEQ ID NO:19), the deduced amino acid sequence of its gene product Env, and neutralizing regions of Env have been disclosed previously (Stewart et al., 1986, *J. Virol*. 58:825-834; Elder et al., 1987, *J. Virol*. 61:8-15, respectively; the disclosures of which are herein incorporated by reference). Cats immunized with the vaccine resisted subsequent challenge with FeLV, in the absence of detectable FeLV-neutralizing antibodies.

Using the methods of the present invention, as illustrated in Examples 1-4, and 7, an expression cassette can be constructed to include any combination of two or more genes, wherein one of the genes encodes the FeLV Env protein. Other exogenous genes that can be included in such an expression cassette include genes that encode FIV Gag, FIV Env, FIPV M, FIPV N, FCV capsid protein, FPV VP2, or rabies-G. The expression cassette may then be inserted into an insertion vector, and subsequently recombined into the TK gene of raccoon poxvirus, thereby forming a multivalent recombinant raccoon poxvirus.

Alternatively, using the methods of the present invention, as illustrated in Examples 1-4, 6, and 7, an expression cassette can be constructed to include any combination of two or more genes, wherein one of the genes encodes FELV Env. Other exogenous genes that can be included in such an expression cassette include genes that encode FIV Gag, FIV Env, FIPV M, FIPV N, FCV capsid protein, FPV VP2, or rabies-G. The expression cassette may then be inserted into an insertion vector, and subsequently recombined into the HA gene of raccoon poxvirus, thereby forming a multivalent recombinant raccoon poxvirus.

In another embodiment, using the methods according to the present invention, both the HA gene and the TK gene can be used as sites into which exogenous genes encoding feline pathogen antigens are recombined into raccoon poxvirus. Combinations of such exogenous genes include genes which encode FeLV Env, FIV Gag, FIV Env, FIPV M, FIPV N, FCV capsid protein, FPV VP2, and rabies-G. With all of these embodiments, using the methods according to Example 5 and other methods known in the art, vaccine stocks of recombinant raccoon poxvirus containing exogenous genes encoding feline pathogen antigens can be prepared, and administered in a vaccination process to felines.

It should be understood that while the invention has been described in detail herein, the examples were for illustrative purposes only. Other modifications of the embodiments of the present invention that are obvious to those skilled in the art of molecular biology, veterinary medicine, and related disciplines are intended to be within the scope of the appended claims.